



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> :  A61K 31/19, 37/04		A1	(11) International Publication Number: <b>WO 90/11071</b>  (43) International Publication Date: 4 October 1990 (04.10.90)
(21) International Application Number: PCT/US90/01567 (22) International Filing Date: 22 March 1990 (22.03.90) (30) Priority data: 327,314                      22 March 1989 (22.03.89)                      US (60) Parent Application or Grant (63) Related by Continuation US    327,314 (CIP) Filed on                                      22 March 1989 (22.03.89) (71) Applicants (for all designated States except US): DANA FARBER CANCER INSTITUTE [US/US]; 44 Binney Street, Boston, MA 02115 (US). THE PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; Cambridge, MA 02138 (US).		(72) Inventors; and (75) Inventors/Applicants (for US only): SPIEGELMAN, Bruce, M. [US/US]; 41 Oakmont Road, Newton, MA 02159 (US). CASTELLOT, John, J., Jr. [US/US]; 3 Albion Place, Newton, MA 02159 (US). DOBSON, Deborah, E. [US/US]; 37 Neillian Crescent, Jamaica Plains, MA 02130 (US).  (74) Agents: MURASHIGE, Kate, H. et al.; Irell & Manella, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).  (81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.  Published <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: ANGIOGENIC BUTYRIC ACID ANALOGS AND THEIR PRODRUGS			
<div style="text-align: center;"> <math display="block">  \begin{array}{ccccccc}  &amp; &amp; X_4 &amp; &amp; X_3 &amp; &amp; X_2 \\  &amp; &amp;   &amp; &amp;   &amp; &amp;   \\  X_4 - &amp; C &amp; - &amp; C &amp; - &amp; C &amp; - COOH \quad (1) \\  &amp;   &amp; &amp;   &amp; &amp;   \\  &amp; X_4 &amp; &amp; X_3 &amp; &amp; X_2  \end{array}  </math> </div>			
(57) Abstract  Butyric acid and its analogs of formula (1) or the physiological salts thereof, wherein each X <sub>2</sub> , X <sub>3</sub> and X <sub>4</sub> is independently H, OH, OR, SH, SR, NH <sub>2</sub> , NHR, NR <sub>2</sub> , or halo, wherein each R is independently lower alkyl(1-4C); or one or two of X <sub>3</sub> and X <sub>4</sub> taken together form a pi-bond, or wherein one of X <sub>2</sub> and X <sub>3</sub> taken together form a pi-bond; with the proviso that at least four of said X must be either H or a participant in a pi-bond, or a prodrug which generates said compound of formula (1) or its salt, are capable of stimulating angiogenesis <i>in vivo</i> . These compounds, and their pharmaceutical compositions, are therefore useful in wound healing and other therapeutic applications where stimulation of vascularization is beneficial. Antibodies to these materials have also been prepared and are useful in diagnosis and therapy.			

### DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

#### *FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Faso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic	KR	Republic of Korea	SN	Senegal
CG	Congo	LJ	Liechtenstein	SU	Soviet Union
CH	Switzerland	LK	Sri Lanka	TD	Chad
CM	Cameroon	LU	Luxembourg	TG	Togo
DE	Germany, Federal Republic of	MC	Monaco	US	United States of America
DK	Denmark				

-1-

10

ANGIOGENIC BUTYRIC ACID ANALOGS AND THEIR PRODRUGSTechnical Field

15           The invention is related to therapeutic compositions for stimulating vascularization in warm-blooded animals. In particular, the invention concerns monobutyric acid and its analogs and their prodrugs which are effective in stimulating angiogenesis.

20

Background Art

          "Angiogenesis" refers to the growth of new blood vessels, or "neovascularization", and involves the growth of capillaries composed of endothelial cells. Angio-  
25   genesis is an integral part of many important biological processes, including cancer cell proliferation, inflammation, wound healing, the menstrual cycle, and fetal development. New blood vessel formation is required for the development of any new tissue, whether normal or  
30   pathological, and thus represents a potential control point in regulating many disease states, as well as a therapeutic opportunity for encouragement of the growth of normal tissue.

          One paradigm for this process is encountered  
35   early in embryonic development when primordial cells form

"blood islands," the cells of which give rise to capillaries, which in turn form the larger veins and arteries in the process of vasculogenesis. However, once the vascular system is formed, usually by the end of the first  
5 third of embryonic development, new capillaries, including those which arise later in the embryo and in the adult, are derived from existing vessels. It is this angiogenesis or neovascularization phenomenon to which the invention is applied.

10 The complete process of angiogenesis is not entirely understood, but it is known to involve the endothelial cells of the capillaries in the following ways:

1) The attachment between the endothelial cells and the surrounding extracellular matrix is altered,  
15 presumably mediated by proteases and glycosidases which may be produced at enhanced levels during this process;

2) There is a "chemotactic" or motility-stimulating process that encourages migration of the endothelial cells toward the tissue to be vascularized; and

20 3) There is a "mitogenesis" process--i.e., proliferation of the endothelial cells to provide additional cells for the new vessels.

Each of these activities involved in angiogenesis: proteolysis, motility-stimulation, and  
25 mitogenesis, can be measured independently in in vitro endothelial cell cultures. In addition, the overall angiogenic stimulation of a test substance can be measured in model systems such as the chick chorioallantoic system (which measures angiogenic activity in an embryonic  
30 system), and in the rabbit corneal pocket assay and in hamster cheek pouch assay (which measure angiogenic activity in more mature systems).

A number of factors are known to stimulate angiogenesis. Many of these are peptide factors, and the  
35 most notable among these are the fibroblast growth factors, both acidic and basic, which can be isolated from

a variety of tissues including brain, pituitary, and cartilage. A discussion of various peptide factors involved in angiogenesis, including acidic and basic FGF is found in, for example, PCT application WO87/01728 published 26 March 1987 and incorporated herein by reference. Both isolated and recombinant forms of many of these proteins are now available.

Other factors which are known to show angiogenic-stimulating activity, but which are not proteins, include prostaglandins E1 and E2 (Ben-Ezra, D., et al., Am J Opthamol (1978) 86:445-461) fragments of hyaluronic acid (West, D.C., et al., Science (1985) 228:1324-1326) and nicotinamide (Kull, F.C., et al., Science (1987) 236:843-845).

A series of papers published by groups from Boston University and Angio Medical Corporation led by N. Catsimpoolas, describe the isolation of a lipid angiogenic factor from omentum. A preliminary communication appeared in JAMA (1984) 252:2034-2036. There are also a number of patent publications from this group, including PCT applications WO87/03804, WO87/03811 and WO87/03812, all published 2 July 1987; WO87/03486, published 18 June 1987; WO87/06136, published 22 October 1987; and U.S. patent 4,699,788, issued 13 October 1987; 4,767,746, issued 30 August 1988; 4,778,787, issued 10 October 1988; and 4,829,114, issued 7 November 1989. These applications describe and claim use of compositions of omental lipids or fractions for skin care and cosmetics or for angiogenesis and epithelialization.

In addition, conditioned media from 3T3 adipocytes, but not from undifferentiated 3T3 cells, has been shown to stimulate angiogenesis related processes in endothelial cells (Castellot, J.J., Jr., et al., Proc Natl Acad Sci USA (1980) 77:6007-6001). This paper described mitogenic activity for endothelial cells in vitro exhibited by the 3T3 adipocyte culture medium which was

insensitive to proteases, not inactivated by heat, and dialyzable.

Further work by this same group, which includes the inventors herein, showed that these conditioned media  
5 (but not the medium conditioned by preadipocytes) could stimulate angiogenesis in the chicken chorioallantoic membrane (CAM) model, and that this stimulation was potentiated by heparin. (Castellot, J.J., Jr., et al., Proc Natl Acad Sci USA (1982) 79:5597-5601). The conditioned  
10 medium was also shown to effect motility stimulation in the Boyden chamber assay described by Zigmond, S., et al., J Exp Med (1973) 137:387-410; Postlethwaite, A.E., et al., J Exp Med (1976) 144:1188-1203; and by Grotendorst, G.R., et al., Proc Natl Acad Sci USA (1981) 78:369-372, all  
15 incorporated herein by reference.

Further characterization of the heparin potentiation of the adipocyte-stimulated angiogenesis was reported by the same group (Castellot, J.J., Jr., et al. (1986) 127:323-329. This work showed that both anti-  
20 coagulant and nonanticoagulant heparin were capable of potentiation of the activity of the 3T3 adipocyte-conditioned media in the CAM assay.

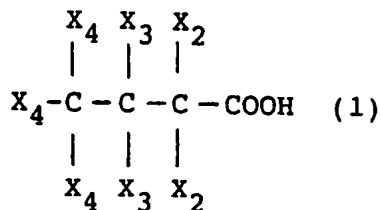
When the three aspects of angiogenesis separable in vitro were tested, it was found that heparin potenti-  
25 ated the effect of the conditioned media on protease activity (measured as plasminogen activator activity) and motility but did not affect the mitogenic activity of the medium. The effect of heparin on the protease activity depended on the nature of the protease--heparin stimulated  
30 tissue plasminogen activator activity, but inhibited urokinase activity.

The present invention provides purified and synthetic factors capable of angiogenesis which are suitable for pharmaceutical formulation and administration.  
35 These factors are members of a group of compounds which are analogs of low molecular weight monoglycerides.

Disclosure of the Invention

The angiogenic compounds of the invention are represented by the formula:

5



10

or the physiological salts thereof, wherein each  $X_2$ ,  $X_3$  and  $X_4$  is independently H, OH, OR, SH, SR,  $NH_2$ , NHR,  $NR_2$ , or halo, wherein each R is independently lower alkyl(1-4C); or one or two of  $X_3$  and  $X_4$  taken together  
 15 form a pi-bond, or wherein one of  $X_2$  and  $X_3$  taken together form a pi-bond;

with the proviso that at least four of said X must be either H or a participant in a pi-bond,  
 20 or a prodrug which generates said amount of the compound of the formula 1 or its salt.

The angiogenic activity of this group of compounds is synergistic with additional growth factors, such as motility-stimulating factors and developmental-morphogenesis factors.  
 25

Accordingly, in one aspect, the invention is directed to pharmaceutical compositions for angiogenesis stimulation, and methods to stimulate angiogenesis which employ the compounds of formula 1 or their prodrugs or mixtures thereof either alone, or in combination with  
 30 peptide growth factors having angiogenesis-promoting activities.

In another aspect, the invention is directed to antisera or individual antibodies immunoreactive with the angiogenic compounds of the invention, to methods to  
 35

assess the level of angiogenic compounds using these antibodies, and to methods to arrest vascularization by passive immunotherapy.

5

#### Brief Description of the Drawings

Figure 1 shows the angiogenesis, chemotaxis, and mitogenesis activity of column fractions from adipocyte-conditioned medium.

10

Figure 2 shows the results of thin-layer chromatography of labeled materials contained in control media or conditioned media when adipocyte cells are labeled with 14-C arachidonate or 14-C acetate.

15

Figure 3 shows the HPLC profile of 14-C acetate-labeled material purified by TLC from 3T3 adipocytes.

Figure 4 shows the biological activity in angiogenesis, chemotaxis, and protease activity of purified monobutyrim.

20

Figure 5 shows an HPLC trace of vacuum-distilled commercially available practical grade monobutyrim.

Figure 6 shows the effect on wound healing of monobutyrim and bFGF separately.

Figure 7 shows the effect of monobutyrim and bFGF separately and together on wound healing.

25

#### Modes of Carrying Out the Invention

30

The invention uses butyric acid and its analogs and their prodrugs as angiogenic factors, with or without additional active components. Monobutyrim, which can be considered a prodrug for butyric acid, was initially isolated from conditioned media.

#### Preparation and Characterization of an Angiogenic Factor from 3T3 Conditioned Medium

35

The conditioned medium of differentiated 3T3 adipocytes is separated into hydrophilic and hydrophobic fractions using any convenient means, but typically using



a reverse phase chromatography method. The hydrophilic fraction, as noted above, is capable of stimulating mitogenesis. The lipid soluble material, which is retained on a reverse phase column, is angiogenic but not mitogenic. An illustrative separation of these materials is as follows:

Dishes (100 mm) of 3T3-F442A adipocytes are covered with 5 ml serum-free Dulbecco's modified Eagle's medium for 24 hr. (If desired, in order to characterize the lipid-like angiogenic factor, a small amount of labeled precursor may be introduced during conditioning.) The adipocyte-conditioned medium is then passed over C18 Sep-Pak columns, washed extensively with phosphate buffered saline (PBS) and water and then eluted with increasing concentrations of aqueous ethanol. The flowthrough and the eluate fractions can be assayed using the CAM assay for angiogenesis as described below, for motility-stimulation activity using Boyden chambers (as described above), and for mitogenesis using bovine aortic endothelial cells (BAEC cells) as described by Castellot, J.J., Jr., et al. Proc Natl Acad Sci USA (1980) 77:6007-6011, cited above and incorporated herein by reference.

Figure 1 shows the elution pattern of a typical column. In this illustrative example, 20 ml of 3T3 adipocyte-conditioned medium was applied to 2 Sep-Pak C18 columns and fractions were eluted in 2 ml portions with the indicated percentages of aqueous ethanol, evaporated to dryness and resuspended in 2 ml control medium containing 0.2% ethanol for assay.

The top frame of Figure 1 shows the results of the CAM test for angiogenesis, which is reported as the percentage of positive responses, when at least 40 samples were tested for each fraction. As shown, the angiogenesis activity occurs in the eluate fractions representing 30 and 50% ethanol.

-8-

The second pattern in the middle frame of Figure 1 represents the results of motility-stimulation assay reported as the net cells/mm<sup>2</sup> on each medium test filter minus the number of cells/mm<sup>2</sup> on a control medium filter. As shown in this elution pattern, the motility-stimulation pattern tracks angiogenesis.

Mitogenesis is reported as the number of cells in the test medium minus the number of cells in control medium, and is shown in the third tier of Figure 1. The mitogenic activity is confined to the flowthrough volume, and does not appear in the eluate.

The angiogenesis-stimulating fractions in the hydrophobic eluate were further characterized by labeling the cells with 50 uCi 14-C acetate or 14-C arachidonate for 16 hr and subjecting the 30-50% ethanol fractions resulting from C18 chromatography of the media to thin-layer chromatography (TLC) on silica gel thin-layer plates (20 x 20 cm, 19 channels with preabsorbent zone, J.T. Baker Company). The TLC plates were developed using the organic phase of ethylacetate:2,2,4-trimethylpentane:acetic acid:water (110:50:20:100 by volume) with the results of radioautography shown in Figure 2.

After radioautography, the thin-layer plate was divided into 8 fractions and eluted with absolute ethanol. For each eluate, 25% was evaporated, resuspended in 0.5 ml control medium (0.5% fetal calf serum in DME) containing 0.2% ethanol and tested for angiogenic activity. Samples were used at a concentration equivalent to that of the starting conditioned medium or were further diluted 1:1 with control medium. Control medium alone yielded 11% positives, and all data are derived from at least 40 samples.

The pattern of angiogenic activity is shown in Table 1 below:

35

-9-

Table 1

	<u>Fraction</u>	<u>Percentage Positive</u>	
		<u>Neat</u>	<u>1:1</u>
5	8	0	--
	7	0	--
	6	22	--
	5	88	87
	4	67	38
10	3	25	--
	2	12	--
	1	11	--

It is clear that the majority of the activity is in fraction 5, with a little remaining in fraction 4. As shown in Figure 2, these positions (indicated by the arrows) do not correspond to materials labeled as a result of culturing with labeled arachidonate; however, the cells labeled with 14-C acetate show a labeled band in the position of angiogenic activity which is found only in the conditioned medium, and not in the control. The activity in this band was confirmed by removal of the acetate-labeled band specifically and subjecting the band to the CAM angiogenesis assay. The results were comparable to those exhibited by adipocyte-conditioned medium: the TLC band provided neat gave 85% positive responses in the assay; the adipocyte-conditioned medium provided neat gave 89%; the TLC band diluted 1:49 gave 40% positive responses while similar dilution of the adipocyte-conditioned medium showed 60% positive responses.

In order to characterize the acetate-labeled material more carefully, 100 ml of the conditioned medium (containing 5 ml 14-C acetate-labeled conditioned medium) was loaded onto a Sep-Pak C18 resin and eluted batchwise with 50% aqueous ethanol. The 50% ethanol sample was then

-10-

dried, loaded onto a silica gel TLC plate and chromatographed in the organic phase of isooctane:ethylacetate:water (50:10:100). The labeled TLC band was visualized by autoradiography, and a region containing the labeled material was scraped, eluted in absolute ethanol, dried, and resuspended in 0.1 ml water. The suspension was subjected to HPLC on a C18 column (Vydak 218.TP) with Waters Guard-pak Bond-pak using a 0-15% aqueous isopropanol gradient over 30 min (flow rate at 1 ml/min). The column was washed with 60% aqueous isopropanol for 40 min. One ml fractions were collected and a 10 ul sample of each fraction was removed and radioactivity determined by liquid scintillation counting. As shown in Figure 3, most of the activity appears in fraction 8 and amounts to an overall recovery of 90% of the counts.

The purified fraction in Figure 3, prepared in a scale-up of the purification described above using 100 ml of the conditioned medium, was subjected to analysis by GC/MS, using electron impact MS, leading to a tentative identification of the peak as comprising mainly monobutyryn--i.e., wherein the compound of formula 1 is esterified to glycerol and all X are H. Synthetic monobutyryn yielded identical analytical data and was tested in the bioassays described in detail below to confirm that the activity contained in the purified material is in fact due to this molecule rather than to an "impurity." The activity pattern for synthetic monobutyryn was virtually identical to that obtained for the purified conditioned medium. These results are shown in Figure 4.

When purified synthetic monobutyryn was tested in the CAM assay, shown in Panel A of Figure 4, the results, expressed as percent positive responses from at least 40 samples, showed that 80% positive responses were obtained at about 34 ng/pellet.

Panel B shows that a maximal response, expressed in cells/mm<sup>2</sup> on polycarbonate filters, was obtained at about 34 ng/ml when purified monobutylin was tested for chemotactic activity.

5 Panel C shows that plasminogen activator activity, expressed as plasminogen-dependent cpm cleaved from the surface of 24 well cluster dishes coated with 3-H fibrin (31,400 cpm), peaked in the same range; in this case synthetic, but unpurified, monobutylin was used in  
10 the assay. These results lead to estimates of ED<sub>50</sub> for motility-stimulating activity of  $1 \times 10^{-9}$  M. For the unpurified material, an apparent ED<sub>50</sub> for plasminogen activator-stimulating activity of 5 ng/ml or  $3 \times 10^{-8}$  M was obtained. Further approximate calculations based on  
15 the specific activity obtained in the fraction labeled with C14 acetate provides an estimate that under the foregoing growth conditions, adipocyte-conditioned medium contains approximately  $6 \times 10^{-8}$  M monobutylin and the activity obtained for the adipocyte-conditioned medium is  
20 thus in good agreement with the activity obtained for monobutylin.

Monobutylin at a range of 3.4 pg/ml-3.4 ug/ml was inactive as a mitogen, as was the 30-50% ethanol eluate of the C18 column run on conditioned medium, as  
25 shown in panel D. The synthetic material at a range of 3.4 pg/ml-3.4 ug/ml was unable to stimulate growth of endothelial cells when added to serum in the range of 0.5-10%.

### 30 Preparation of Compounds Useful in the Invention

The compounds of formula 1 useful in the invention method comprise butyric acid, butenoic acid, and the simple substituted analogs thereof. Accordingly, the preparation of these compounds is straightforward. Alpha-  
35 substituted butyric acid, such alpha-aminobutyric and

alpha-hydroxybutyric acid are readily available. Accordingly, additional substitutions at the alpha position are readily made. Substitutions at the beta and gamma positions are easily accomplished by addition to the  
5 pi-bond in 3,4-butenic acid.

The compounds useful in the invention in prodrug form are generally derivatives of the carboxylic acid functionality. Suitable prodrugs are amides, imides, esters, anhydrides, thioesters or thioanhydrides involving  
10 this functional group. Methods for preparation of these compounds are well understood in the art. Particularly preferred prodrugs are the monoesters of the compound of formula 1 with glycerol; these prodrugs can be prepared conventionally by esterification with glycerol and purifi-  
15 cation of the monoesterified product. Isolation of the monoester can be done by any standard means, such as chromatographic separation or fractional distillation. The ester and amide prodrugs are readily prepared from the corresponding carboxylic acid of formula 1 or their acti-  
20 vated (acyl halide or anhydride, for instance) derivatives and glycerol or other alcohol or amine using standard esterification methods. Purification of the resulting monoesters (or diesters or triesters) is accomplished by chromatographic methods, e.g., HPLC, GC, or other standard  
25 preparative methods.

Some of the compounds of formula 1 and their prodrugs contain at least one chiral center and thus can be prepared in optically active form or as racemic mixtures. Unless otherwise specified, the compounds of  
30 the invention are racemic, or, in the case of the forms isolated from biological systems, of the naturally occurring stereoisomer. Individual stereoisomers are, however, generally included within the scope of the invention.

35

For purposes of illustration, a protocol for the purification of commercially available monobutyryn to a purity level of greater than 99% is as follows:

Practical grade monobutyryn having a boiling point of 153°C is subjected to vacuum distillation at about 3 mm Hg over an oil bath held at 160-162°C and the distillate obtained at 137-138°C is recovered. This fraction is subjected to chromatography through a 50 cm x 1.5 cm column containing 30 g of silica gel, and eluted by gradient elution with hexane:ethylacetate varying from 90:10-20:80 v/v, collecting 10 ml fractions. In one typical run, fractions 29-33 contained the butyryn. The AGC chromatogram (DB-1 column) run on these fractions is shown in Figure 5. The first peak eluted from this chromatography, when obtained preparatively, is recrystallized from ether at -70°C. In a typical procedure, this material was shown to be more than 99% pure by GC and TLC, and confirmed by mass spectrometry to be monobutyryn.

Similar purification procedures can be used for commercial or synthetic preparations of this and alternate embodiments of the compound of formula 1.

#### Preferred Embodiments

Preferred prodrugs include amides, amides, esters, anhydrides, thioesters, and thioanhydrides of the carboxy group of the compound of formula 1, preferably the glyceryl esters and most preferably the monoglyceryl ester.

Other suitable alcohols with which esters may be formed include lower alkyl alcohols such as methyl, ethyl, or isobutyl alcohol; other polyhydroxy alcohols such as ethylene glycol; polymeric alcohols which are relatively hydrophilic, such as polyethylene glycol or polyvinyl alcohol; and substituted alcohols such as 2-methoxy-ethanol. Thioesters are formed from the corresponding sulfhydryl-containing compounds. Suitable amines include

lower alkyl primary and secondary amines such as ethylamine, diethylamine, methylethylamine, and the like, as well as polymers such as polyallylamine or substituted amines such as aminoethanol.

5           When polymeric alcohols or amines are used as the esterifying or amide-forming groups, multiplicities of the butyric acid analog can be coupled to the polymeric carrier.

10           In general, among preferred embodiments are those which constitute the alkyl(1-8C) or aryl(1-8C) esters, thioesters or amides of the compound of formula 1.

          As used herein, "alkyl(1-8C)" refers to a straight chain or branched chain or cyclic saturated hydrocarbyl residue, which may contain one or two  
15 nonhydrocarbyl substituents which do not interfere with or alter the functionality of the hydrocarbyl moiety. Examples of such alkyl(1-8C) moieties include straight chain hydrocarbyl of the formula  $-(CH_2)_nCH_3$  wherein n is an integer of 0-7; branched chain residues such as  
20 isobutyl, isohexyl, tertiary butyl; and cyclic residues such as cyclopentyl, cyclohexyl, methylcyclopentyl, metholcyclohexyl, and the like. Alkyl acyl(1-8C) refers to RCO wherein R is alkyl(1-7C) as above defined. Aryl acyl refers to RCO wherein R is aryl(1-7C) as defined  
25 below.

          As used herein, "lower alkyl(1-4C)" refers to a straight chain or branched chain saturated hydrocarbyl residue, which may contain one or two nonhydrocarbyl substituents which do not interfere with or alter the  
30 functionality of the hydrocarbyl moiety. Examples of such lower alkyl(1-4C) moieties include methyl, ethyl, isobutyl and tertiary butyl.

          "Aryl(1-8C)" refers to substituted or unsubstituted phenyl moieties wherein the substituents are halo, methoxy or ethoxy and also includes alkaryl substituents  
35 such as phenylmethyl or phenylethyl.



-15-

Preferred forms of the compounds of formula 1 include:

those wherein one X is OH, OR, SH, SR, NH<sub>2</sub>, NR<sub>2</sub>, F, Cl, or Br, and the rest are H, and

5 those wherein two of X are independently OH, OR, SH, SR, NH<sub>2</sub>, NR<sub>2</sub>, F, Cl, or Br and the other X are H, and

those wherein one or two of X<sub>3</sub> and X<sub>4</sub> are participants in a pi-bond and all other X are H, or

10 those wherein one of X<sub>2</sub> and X<sub>3</sub> taken together from a pi-bond and all other X are H.

Thus, preferred forms of the compound of formula 1 include butyric acid; 2,3-butenic acid; 3,4-butenic acid; alpha-aminobutyric acid; gamma-amino butyric acid; alpha-chlorobutyric acid; gamma-  
15 hydroxybutyric acid; and the like. In embodiments wherein one or more X is a functional group, the prodrug may contain an additional moiety coupled through this functional group. For example, the acyl(1-8C) ester of an alpha, beta or gamma hydroxyl is included in this group.

20 The angiogenic activity of the particular embodiment of the compound of formula 1 or its prodrug considered may be verified using the CAM or diabetic mouse assays described below.

## 25 Assay Methods

A: CAM Assay: Angiogenic activity is conveniently measured by the chick chorioallantoic membrane assay using a modification of the shell-less embryo technique described by Dunn et al., Anat Rec (1981)  
30 199:33-43. In this procedure, chicken eggs, 60-72 hr after fertilization, are cracked into a plastic wrapped sling, held in a styrofoam drinking cup with a rubber band as described by Castellot, J.J., et al., J Cell Phys (1986) 127:323-329. The cup is covered with a sterile  
35 plastic top from a 100 mm culture dish and placed in a humidified 37°C incubator. Nine days after fertilization,

30 ul or 40 ul agarose pellets containing test substances are placed on the chorioallantoic membrane which occupies an area of 30-40 cm<sup>2</sup> at this stage. (Pellets are prepared by rapidly combining 15 or 20 ul of 6% aqueous low melting point agarose (Sigma Type VII) with an equal volume of test substance at 37°C, thereby avoiding heating of the test substance, and the resulting pellets are kept at 4°C for several minutes to solidify.)

Observations are made visually, and 5-8 samples can be placed on a single egg. Vascularization is observed visually, and scoring is done in a single-blind fashion on days 2-5 after adding the pellets. Histological sections of positive responses are examined for the presence of inflammatory cells, since inflammation of the membrane could also result in a positive response, and pellets that have clearly elicited an inflammatory response, as evidenced by the translucent pellet becoming white and opaque, are not scored. More than 50% of the embryos ordinarily survive to permit testing.

Results are given as the percentage of pellets of a test substance which show vascularization, of more than 40 pellets typically tested.

#### B: Diabetic Mouse Model

SPF female diabetic (db/db) mice are obtained from Jackson Labs at 4-5 weeks old and acclimated for several weeks before testing. The animals are anesthetized with methoxyflurane and hair clipped with a no. 40 blade and the surgical site then denuded with a no. 10 scalpel blade. The surgical site is then cleaned with 70% ethanol and dried.

A 1.5 cm diameter circle is marked on the dorsal surface to the left side of the dorsal column and a full thickness incision made using scissors. The area of the wound is 2 cm<sup>2</sup>.

Test substances are applied in a 20 ul volume in multiple aliquots over a one minute period. After absorption of the liquid, the site is covered with a sterile occlusive dressing.

5           The surgical incisions are made on Day 0 and the test solutions are applied at that time. Dressings are changed at 3 day intervals beginning on Day 3 up to Day 24, and the wound areas traced onto acetate sheets to determine areas, using the Jandel Scientific digitizing  
10 program Sigma Scan, at each dressing change. Blood glucose levels are determined once a week, and the animals are weighed twice weekly.

Other Assays: The ability of a test substance  
15 to elicit a response in vitro corresponding to plasminogen activation, motility stimulation, or mitogenesis can also be tested by standard means. Convenient assay systems to conduct these tests are set forth in Castellot, J.J., Jr., J Cell Physiol (1986) 127:323-329, cited above, and  
20 incorporated herein by reference.

#### Effect of Added Materials

          Combination of the angiogenic compounds of the invention of formula 1 or its prodrugs with growth factors  
25 is beneficial. A number of factors, mostly proteinaceous, which enhance the growth of endothelial and other cells have been prepared, and a summary and comparison of 13 of these factors is found in Lobb, R., et al., J Biol Chem (1986) 261:1924-1928, incorporated herein by reference.  
30 Prominent among these factors are platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and the acidic and basic fibroblast growth factors (FGF). These factors, in combination with the compound of formula 1 or its prodrugs, permit more effective stimulation of, for  
35 example, wound healing.

The additional factors may have mitogenic and/or chemotactic activity. Various means of classifying these factors have been suggested. For example, motility-stimulating factors are considered to include basic FGF, Iamin (Gly-His-Lys ( $\text{Cu}^{+2}$ )), and the tripeptide F-Met-Leu-Phe. Neurotrophic factors also include FGF, as well as NGF and CNTF. Developmental-morphogenesis factors include bFGF and TGF-beta.

An alternative classification envisions various families such as the basic FGF family, which includes acidic FGF, int-2, K-FGF(hst), FGF-5, FGF-6 and KGF; the EGF family, which includes TGF-alpha, VGF and amphiregulin; the PDGF family which includes AA, BB and AB forms and VEGF; the TGF-beta family which has six analogous members; the insulin growth factor family which includes at least two analogous members, and the mammalian growth hormone family which includes human and bovine growth hormone.

By including one or more of these growth factors in the compositions and methods of the invention, the amounts of each component can be lowered. Thus, an effective amount of the combination will involve amounts of each member which are less than would be required if the factors were used individually.

As an illustration, various amounts of monobutyryn and basic FGF, as described in WO87/01728, were combined and assayed in the CAM method set forth above. Control pellets were prepared containing only buffer (0.9% NaCl) and elicited only 9% positive responses. As positive controls, basic FGF at 30 ng/pellet yielded 81% positive responses and higher concentrations of monobutyryn at 340 ng/pellet yielded 92% positives. In the assay, monobutyryn at 34 pg/pellet yielded 24% positive responses; basic FGF at 1 ng/pellet yielded 15% positive responses. An additive effect of mixing 34 pg monobutyryn

and 1 ng basic FGF in a pellet would have yielded about 39% positive responses; 72% positives were obtained.

In an additional trial, the CAM assay was conducted using highly purified synthetic monobutyryn, recombinant human basic FGF and their combination, with a buffer control-containing vehicle alone (DMEM + 2% fatty acid-free, immunoglobulin-free bovine serum albumin). A total of at least 30 pellets of each of the samples was tested in two separate experiments.

The results showed that the buffer control elicited only 15% positive responses; 20 pg/pellet monobutyryn elicited 28%; 2 ng/pellet FGF elicited 22%; and a pellet containing both 20 pg monobutyryn and 1 ng FGF elicited 63% positive responses.

These results show that the monobutyryn and basic FGF behaved in a synergistic manner in this assay.

The effects of recombinant human basic FGF and monobutyryn were also investigated in vivo in observing the rate of wound healing in diabetic mice. The assay is described in detail above. The results are shown in Figures 6 and 7.

Figure 6 shows a comparison of various treatments in effecting a decrease in wound area. In Figure 6, the open squares represent a vehicle control; the open triangles show the effect of 1 ug/cm<sup>2</sup> of basic FGF; the closed diamonds show the effect of 1 ug/cm<sup>2</sup> of monobutyryn; the closed triangles represent the effect of 10 ug/cm<sup>2</sup> of monobutyryn. As shown in the figure, all of the treatments show accelerated wound healing over the control.

Figure 7 shows the results for a similar experiment using bFGF and monobutyryn separately and in combination at 0.1 ug/cm<sup>2</sup>. In this figure, the open polygons represent vehicle controls, and the closed polygons represent test substances. As shown in Figure 7, the closed squares representing the application of

0.1 ug/cm<sup>2</sup> of bFGF and the closed triangles, representing the application of 0.1 ug/cm<sup>2</sup> of monobutylin, accelerate wound healing only slightly, with a decrease in area beyond controls which is not significant. However, the  
5 combination of 0.1 ug/cm<sup>2</sup> each of monobutylin and bFGF, as shown in the closed diamonds, gives a significant acceleration in wound healing.

#### Preparation of Antisera

10           The compound of formula 1 may be rendered immunogenic by conjugation to carrier and/or addition of adjuvant and used to prepare antiserum using standard procedures. The resulting antibodies are useful in detecting the levels of these materials in subjects who  
15 have been administered the compounds for therapeutic purposes, as well as for assessing production of the corresponding native factor, and for antiproliferative therapy, for example in controlling the growth of neoplastic tissue.

20           The compound of formula 1 is administered with adjuvant and/or conjugated to a carrier such as keyhole limpet hemocyanin (KLH), tetanus toxoid, or the relevant serum albumin by means known in the art per se, conveniently employing bifunctional linkers such as those  
25 produced by Pierce Chemical Co. The free carboxyl of formula 1 is a convenient functional group for reaction with linkers which can then be conjugated to carrier.

          Immunization is conducted using standard protocols and the antisera obtained and titrated using  
30 standard immunoassay procedures with the relevant compound of formula 1 as antigen. If desired, antibody-producing cells of immunized subjects can be immortalized and screened to obtain monoclonal immunoreactive compositions.

Utility and Administration

In one application, the compounds of the invention having angiogenic activity are useful in encouraging wound healing and are generally applied topically to the traumatized tissue. Appropriate substrates are burns, wounds, dermal ulcers, bone fractures, surgical abrasions or incisions such as those of plastic surgery, damaged neurological tissue, periodontal tissue subjected to trauma, grafted skin, skin flaps, intestinal anastomoses, and organ transplants.

Systemic or internal conditions which require treatment to encourage vascularization include bone fractures, ligament and tendon repair, tendonitis and bursitis, and tissue repair during ischemia and myocardial infarction. The invention compositions may be used for repopulation with endothelial cells of arteries denuded of such cells by balloon angioplasty or by atherosclerotic diseases. Skin conditions such as burns, bed sores and slow-healing ulcers, as well as superficial wounds and deficient hair growth may benefit from topical administration.

A decreased vascularity of the scalp is considered to be a contributing factor to baldness since a good vascular supply is required for healthy hair follicles. Topically administered monobutyryn should penetrate the skin of the scalp and enhance the vascularity of the area containing hair follicles. This would result in the arrest of hair loss.

Formulations of the compounds of the invention are prepared using generally known excipients and carriers according to methods known in the art, such as those set forth in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA (latest edition).

For topical administration, especially for superficial lesions, standard topical formulations are employed using, for example,  $10^{-4}$ -1% solutions of the

compounds of the invention, with or without added growth factor, such as FGF, PDGF or EDGF. The topical preparations would be applied daily or 2-3 times per week to the affected area. The concentration of the solution or  
5 other formulation depends on the severity of the wound and the nature of the subject. Formulations can be in a variety of known forms such as ointments, salves, gels, sprays, creams and lotions.

For bone and tissue repair, local and depot  
10 administration is preferred, or administration by subcutaneous or localized implant. Slow release forms can be included in polymers such as Hydron (Langer, R., et al., Nature (1976) 263:797-799) or Elvax 40P (DuPont) (Murray, J.B., et al., In Vitro (1983) 19:743-747). Other  
15 sustained release systems have been suggested by Hsieh, D.S.T., et al., J Pharm Sci (1983) 72:17-22.

As to dosage levels, in general, topical, systemic and implanted formulations are designed to achieve a constant local concentration of about 100 times  
20 the serum level of the counterpart to the compound of formula 1 or about 10 times the tissue concentration, as described by Buckley et al., Proc Natl Acad Sci USA (1985) 82:7340-7344. As the concentration of the factors of formula 1 appears to be quite low, typically, adminis-  
25 tration of sufficient material to obtain a release of about 10-1000 ng of the compound of formula 1/hr locally is approximately correct. Reduced amounts are needed when the complementary growth factors are included in the composition.

30 It should be noted that as these materials are often used in connection with surgery, it may be helpful to imbed them in polymers used as surgical staples or sutures such as polylactic and glycocholic copolymers.

The antibody preparations immunoreactive with  
35 the compounds of formula 1 useful in the invention may also be formulated into therapeutic dosages for systemic



or local administration using standard administration procedures to control the growth of vascularization in harmful tissues, such as neoplasms or ischemic brain and heart tissue.

5

#### Illustrative Formulations

The following are illustrative of useful formulations in which a compound of formula 1 or its prodrug is active ingredient. For purposes of example, the active ingredient is set forth as monobutyryn in A and B, and as monocaprylin in C. Other compounds of formula 1 or their prodrugs, or mixtures thereof could, of course, be substituted.

#### 15 Formulation A:

A monobutyryn cream is prepared from the following:

	<u>Ingredient</u>	<u>Amount</u>
20	Monobutyryn	0.1 g
	Polysorbate-60	8.0 g
	Cetyl Alcohol	10.0 g
	Myristyl Stearate	5.0 g
	Isopropyl Palmitate	10.0 g
25	Methyl Paraben	0.2 g
	Water q.s.	100.0 g

The cetyl alcohol, myristyl stearate, isopropyl palmitate and polysorbate 60 are heated to about 90°C and melted to obtain an "oil phase".

Most of the required water is heated to 70°C. Methyl paraben is added to the water and stirred until dissolved. Monobutyryn is then added and stirred until dissolved. The resulting aqueous phase is added to the oil phase while both phases are still hot (approximately 60°C) and stirred and homogenized or worked in colloid

mill if necessary. Water is added to make up to 100 g and the mixture is stirred gently until the resulting cream cools.

5 Formulation B:

A monobutyrim gel composition is prepared from the following:

	<u>Ingredient</u>	<u>Amount</u>
10	Monobutyrim	0.1 g
	Hydroxyethylcellulose 250H	2.0 g
	Glycerine	15.0 g
	Chlorhexidine gluconate	0.25 g
	Water q.s.	100.0 g

15

Chlorhexidine gluconate, monobutyrim and glycerine are added to majority of the water and stirred well until complete dissolution occurs. Hydroxyethylcellulose is gradually added and stirred vigorously until all the gelling agents are completely dissolved. Water is then added to 100 g.

20

Formulation C:

An ointment product is prepared as follows:

25

<u>Ingredient</u>	<u>Amount</u>
Monocaprylin	0.1 g
Polyethylene	0.5 g
Heavy Mineral Oil	95.0 g

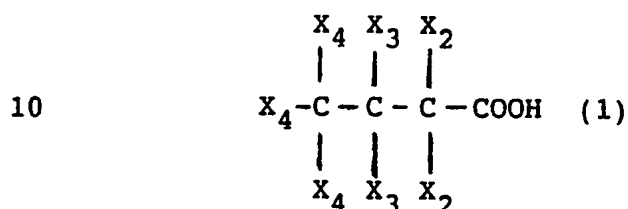
30

The monocaprylin is dissolved in heavy mineral oil and heated to 130°C. The polyethylene is added, and the mixture stirred to dissolve. The mineral oil solution is shock cooled and the gel scraped from the cooling drum.

35

Claims

1. A method to stimulate angiogenesis in a warm-blooded animal, which method comprises administering to a subject in need of such treatment an angiogenically effective amount of an angiogenic compound of the formula:



- or the physiological salts thereof, wherein each  $X_2$ ,  $X_3$  and  $X_4$  is independently H, OH, OR, SH, SR,  $NH_2$ , NHR,  $NR_2$ , or halo, wherein each R is independently lower alkyl(1-4C); or one or two of  $X_3$  and  $X_4$  taken together form a pi-bond, or wherein one of  $X_2$  and  $X_3$  taken together form a pi-bond;

- with the proviso that at least four of said X must be either H or a participant in a pi-bond, or a prodrug which generates said amount of the compound of the formula 1 or its salt.

2. The method of claim 1 wherein said prodrug is an amide, an imide, an ester, an anhydride, a thioester, or a thioanhydride of the carboxy group of the compound of formula 1.

3. The method of claim 2 wherein said prodrug is a glyceryl ester of the compound of formula 1.

4. The method of claim 3 wherein the prodrug is a monoglyceryl ester of the compound of formula 1.

5. The method of claim 1 wherein one of X is OH and the prodrug is the alkyl acyl(1-8C) or aryl acyl(1-8C) ester of the compound of formula 1.

5                   6. The method of claim 1 wherein one of X is NH<sub>2</sub> or NHR and the prodrug is the alkyl acyl(1-8C) or aryl acyl(1-8C) amide of the compound of formula 1.

7. The method of claim 1 wherein one of X is SH  
10 and the prodrug is the alkyl acyl(1-8C) or aryl acyl(1-8C) thioester of the compound of formula 1.

8. The method of claim 1 wherein the prodrug is an alkyl(1-8C) or aryl(1-8C) ester or the anhydride of the  
15 carboxy of formula 1.

9. The method of claim 1 wherein the prodrug is an alkyl(1-8C) or aryl(1-8C) thioester or the thioanhydride of the carboxy of formula 1.

20

10. The method of claim 2 wherein the prodrug is an alkyl(1-8C) or aryl(1-8C) amide or the imide of the carboxy of formula 1.

25                   11. The method of claim 1 wherein one X is OH, OR, SH, SR, NH<sub>2</sub>, NHR, NR<sub>2</sub>, or F, Cl, or Br and wherein the remainder of X are H.

12. The method of claim 1 wherein two X are  
30 independently OH, OR, SH, SR, NH<sub>2</sub>, NHR, NR<sub>2</sub>, or F, Cl, or Br and wherein the remainder of X are H.

13. The method of claim 1 wherein one or two of X<sub>3</sub> and X<sub>4</sub> are participants in a pi-bond and all other X  
35 are H, or wherein one of X<sub>2</sub> and X<sub>3</sub> taken together form a pi-bond and all other X are H.

14. The method of claim 1 wherein the subject requires angiogenesis for wound healing.

5 15. The method of claim 14 wherein said wound is selected from the group consisting of pressure sores, burns, venous ulcers, diabetic ulcers, lacerations and abrasions, surgically or medically induced wounds, skin grafts, skin flaps, fractures, and cardiovascular wounds.

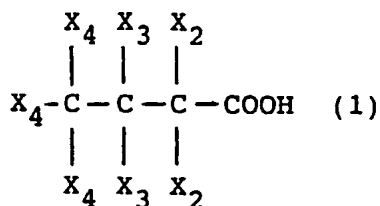
10 16. The method of claim 1 wherein said subject requires angiogenesis for promotion of hair growth.

15 17. A method to stimulate angiogenesis in a warm-blooded animal, which method comprises administering to a subject in need of such treatment an amount of a prodrug which generates an angiogenic compound of formula 1 as defined in claim 1 and an amount of a peptide growth factor, which prodrug and growth factor together are effective to stimulate angiogenesis in said subject.

20 18. The method of claim 17 wherein said growth factor is a member of a growth factor family selected from the group consisting of the fibroblast growth factor family, the epidermal growth factor family, the platelet-derived growth factor family, the transforming growth factor family, the insulin family, the mammalian growth hormone family, the nerve growth factor family, and the CNTF family.

30 19. A pharmaceutical composition suitable for administration to a subject in need of angiogenic stimulation, which composition comprises an angiogenically effective amount of an angiogenic compound of the formula:

35



5

or the physiological salts thereof, wherein each  $X_2$ ,  $X_3$  and  $X_4$  is independently H, OH, OR, SH, SR,  $NH_2$ , NHR,  $NR_2$ , or halo, wherein each R is independently lower  
 10 alkyl(1-4C); or one or two of  $X_3$  and  $X_4$  taken together form a pi-bond, or wherein one of  $X_2$  and  $X_3$  taken together form a pi-bond;

with the proviso that at least four of said X must be either H or a participant in a pi-bond,  
 15 or a prodrug which generates said amount of the compound of the formula 1 or its salts;

in admixture with a pharmaceutically acceptable excipient.

20 20. A pharmaceutical composition suitable for administration to a subject in need of angiogenic stimulation, which composition comprises an amount of a compound of the formula 1 or its salt or a prodrug therefor as defined in claim 1 in combination with an amount of a  
 25 peptide growth factor, wherein said amounts are together effective in stimulating angiogenesis in said subject, said compound of formula 1 or its salt or prodrug therefor and said growth factor in admixture with a pharmaceutically acceptable excipient.

30

21. A method to stimulate motility of endothelial cells which comprises treating said cells with an effective amount of a compound of formula 1 or its salt or a prodrug therefor as defined in claim 1.

35

22. A method to effect protease activity in endothelial cells which comprises treating said cells with an effective amount of a compound of formula 1 or its salt or a prodrug therefor as defined in claim 1.

5

23. Antibodies or antisera immunoreactive with the prodrug of claim 1 or the compound of formula 1 generated therefrom.

10

24. A method to assess the level of angiogenic compound of formula 1 in a biological sample, which method comprises:

contacting the biological sample with antibodies immunoreactive with said compound and

15

detecting the amount of said antibody bound by the sample.

25. A method to assess the level of angiogenic prodrug in a biological sample, which method comprises:

20

contacting the biological sample with antibodies immunoreactive with said prodrug and

detecting the amount of said antibody bound by the sample.

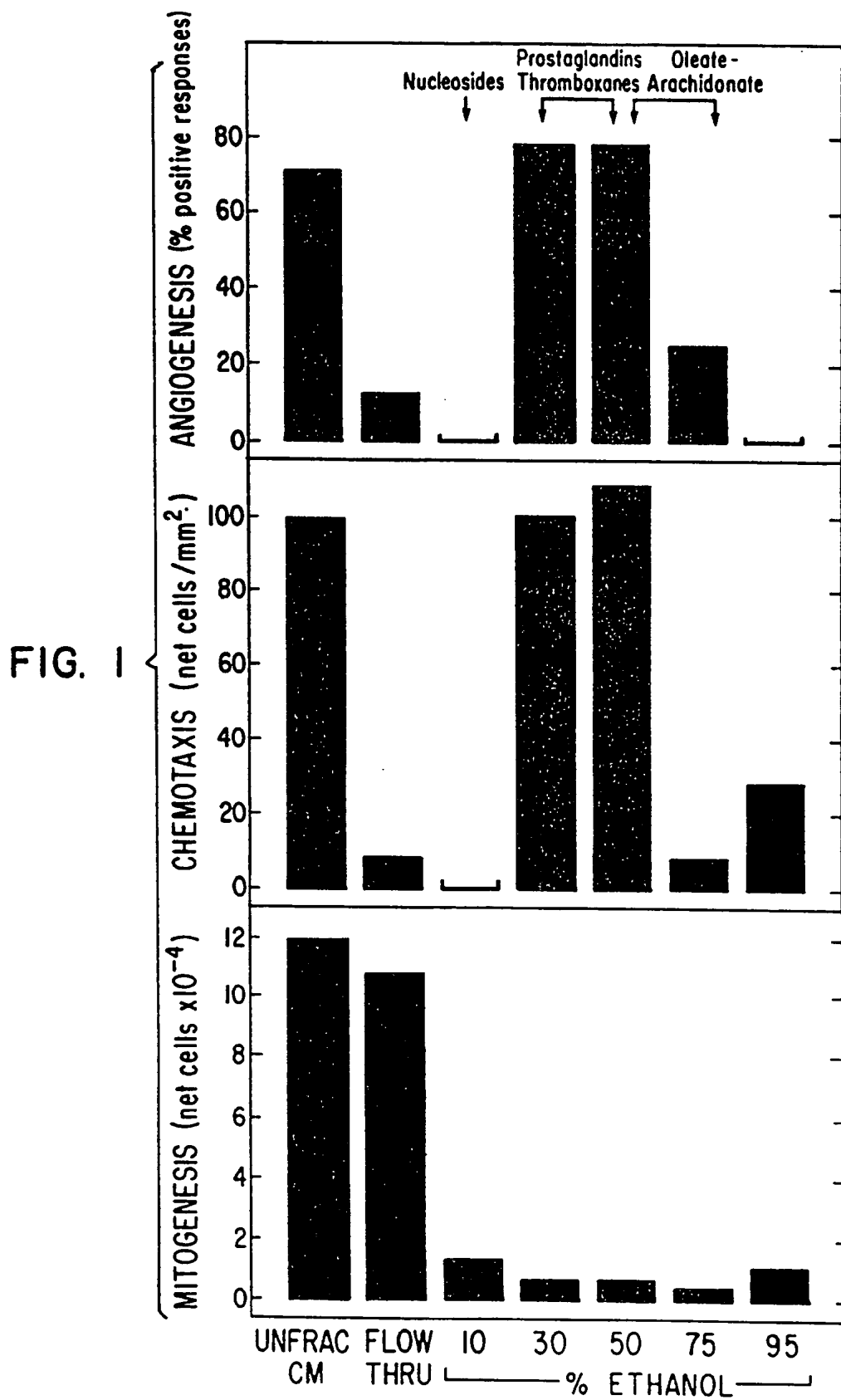
25

26. A method to arrest vascularization in unwanted tissue, which method comprises treating said tissue with an amount of antibodies immunoreactive with the compound of formula 1 or the prodrug thereof effective to arrest said vascularization.

30

35

1 / 7



SUBSTITUTE SHEET



2/7

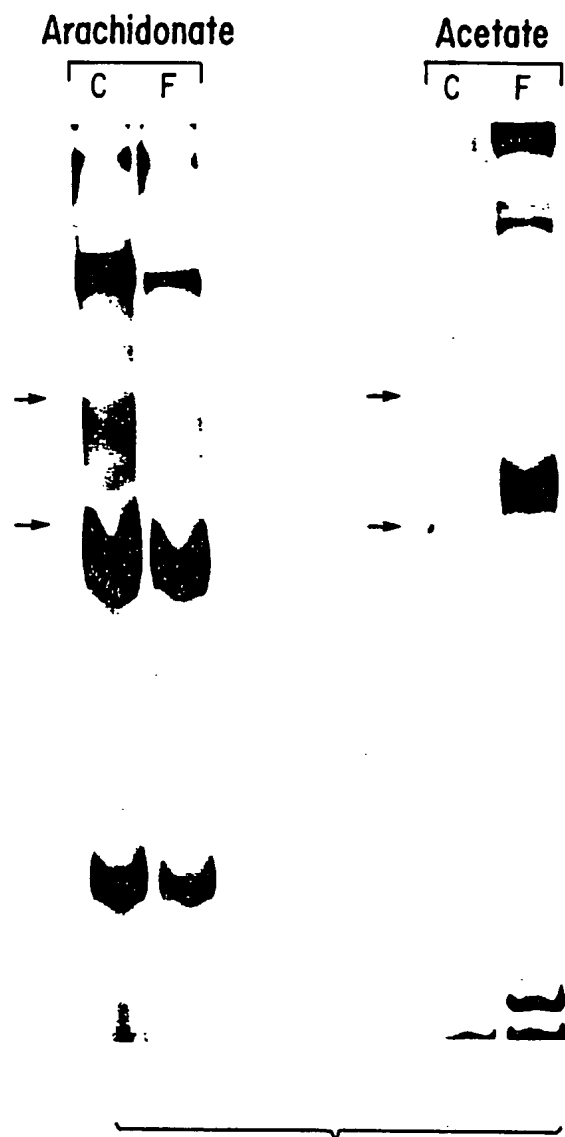


FIG. 2

3/7

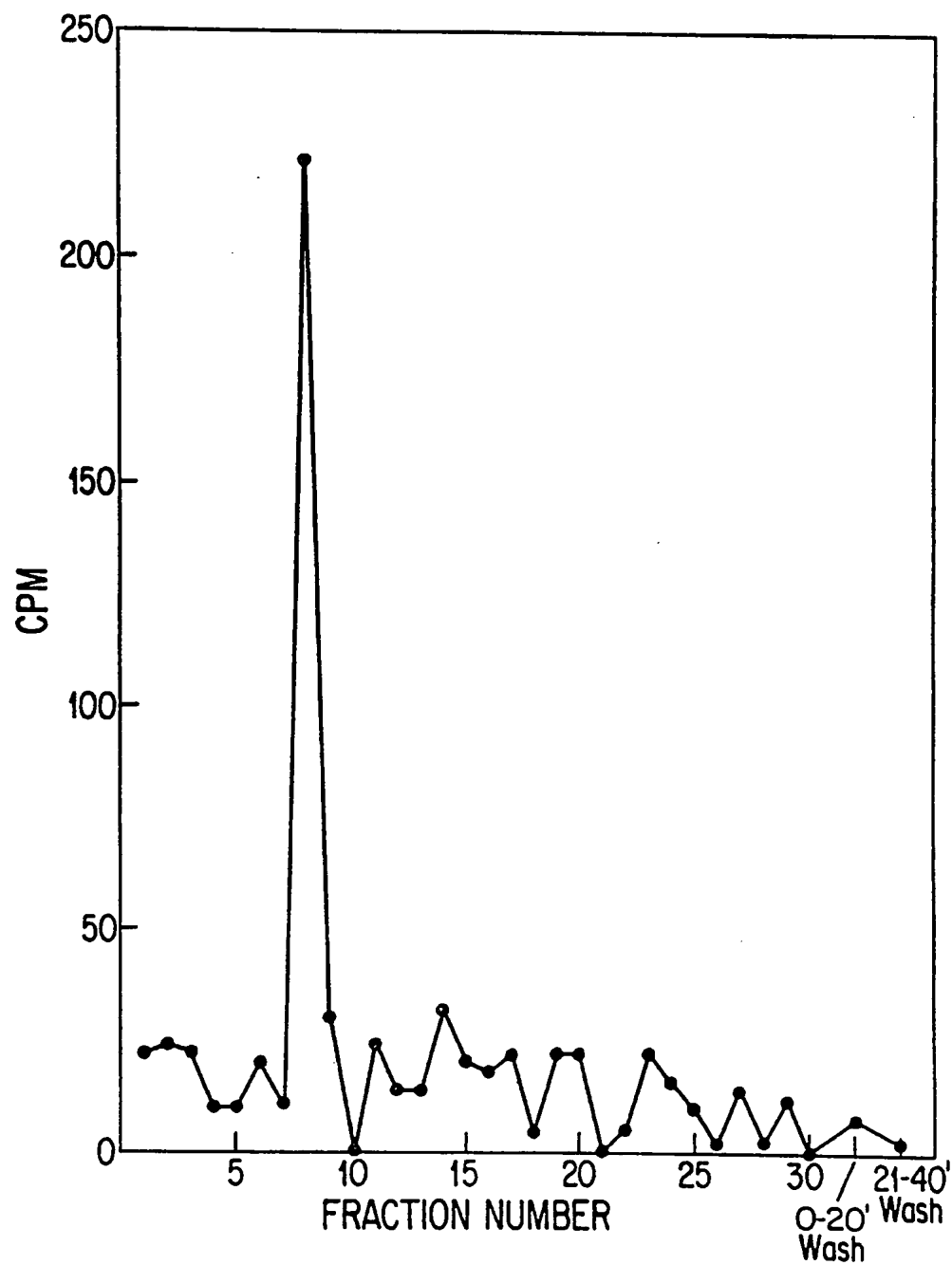


FIG. 3

SUBSTITUTE SHEET

4/7

FIG. 4A.

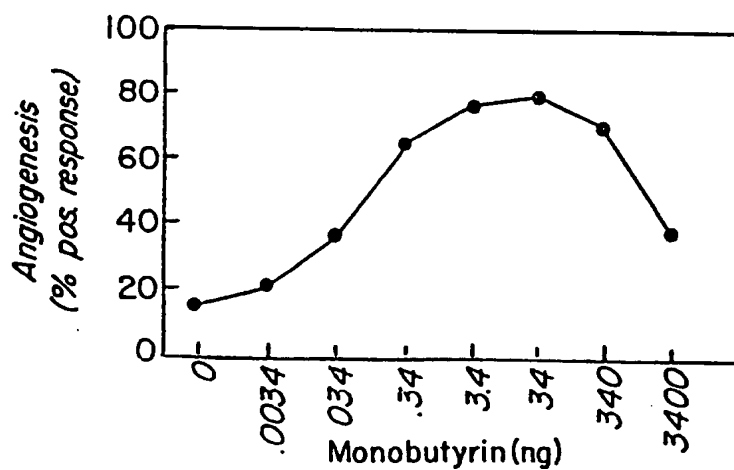


FIG. 4B.

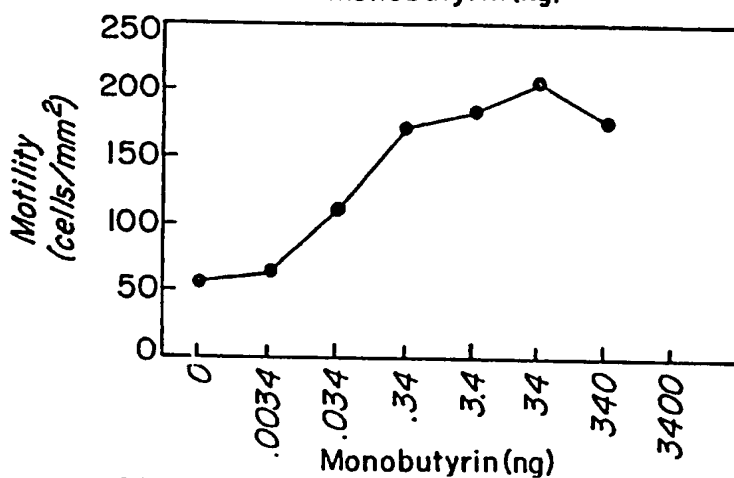


FIG. 4C.

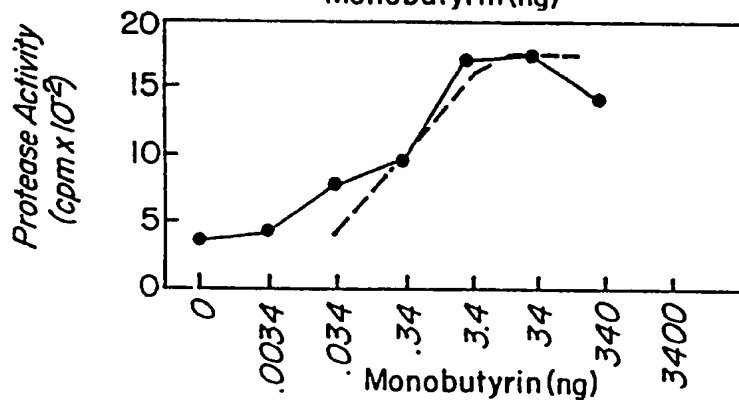
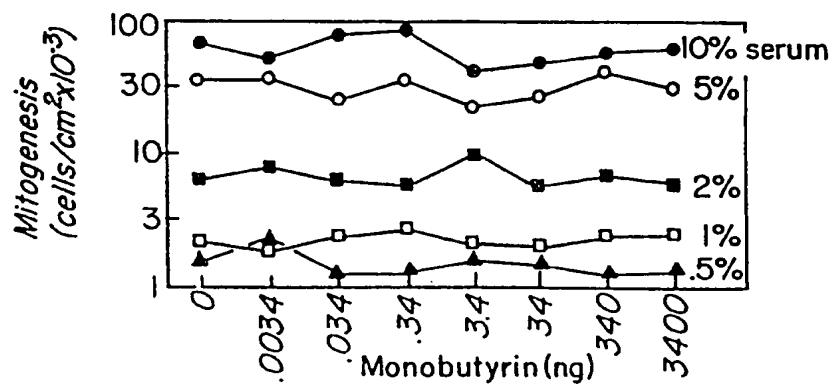


FIG. 4D.



SUBSTITUTE SHEET

5/7

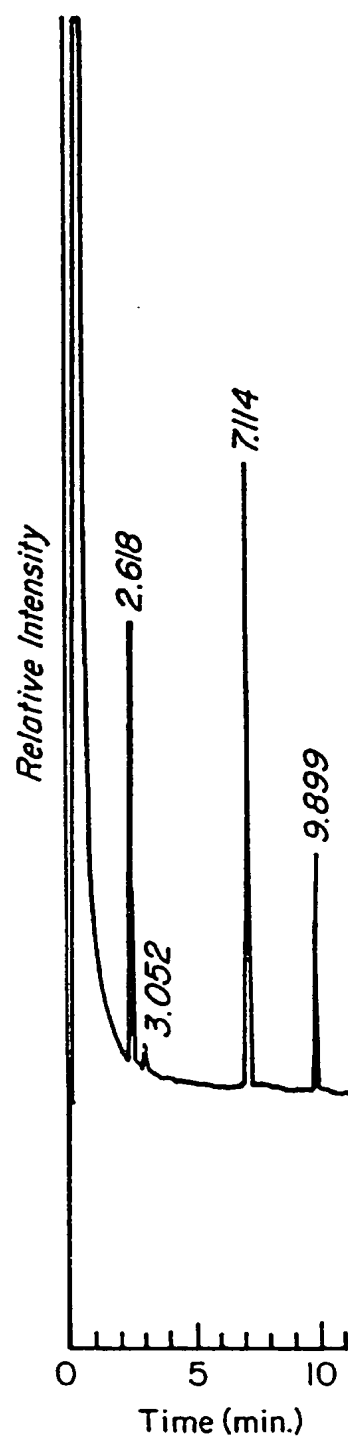


FIG. 5

SUBSTITUTE SHEET

6/7

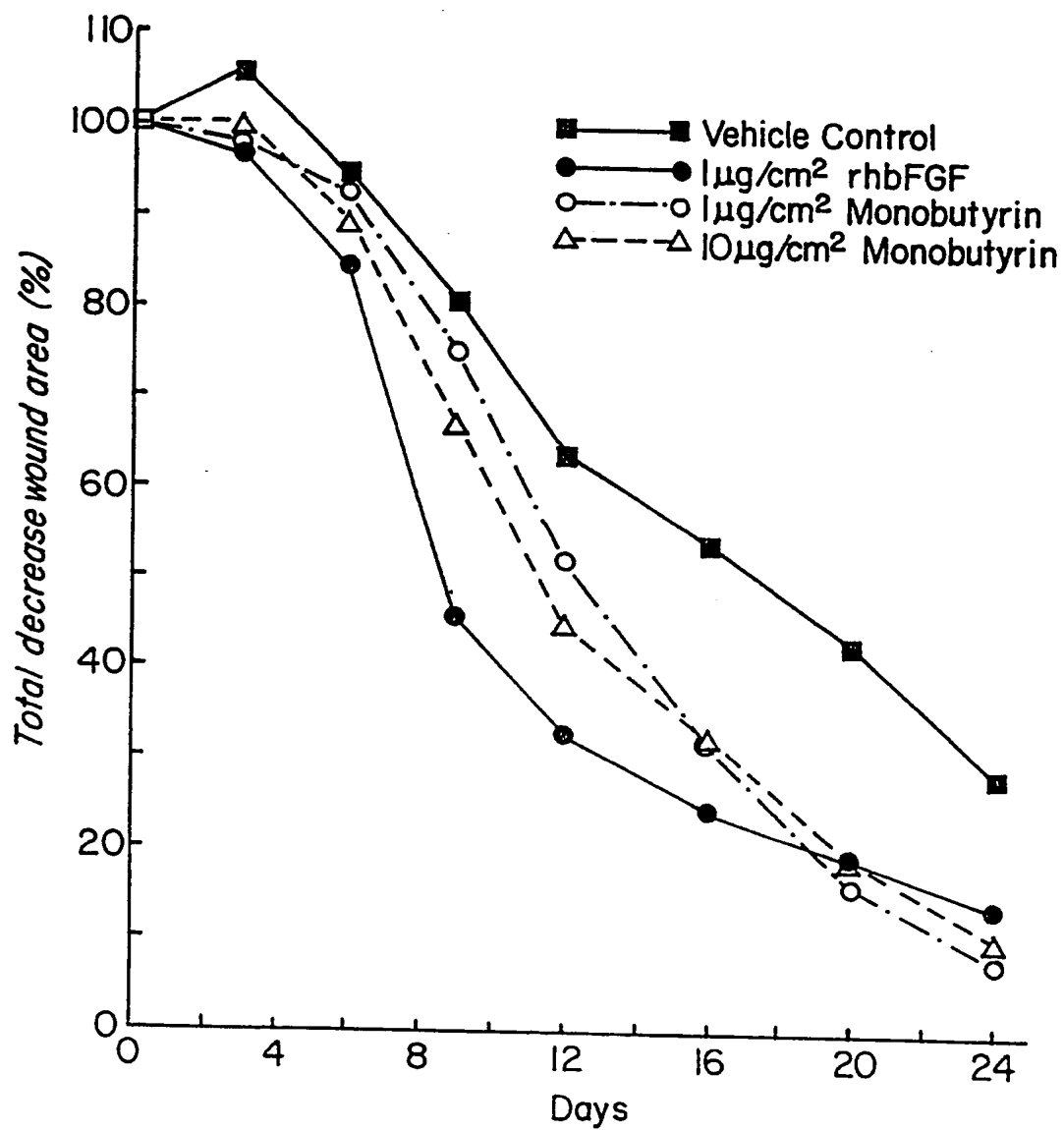


FIG. 6

7/7

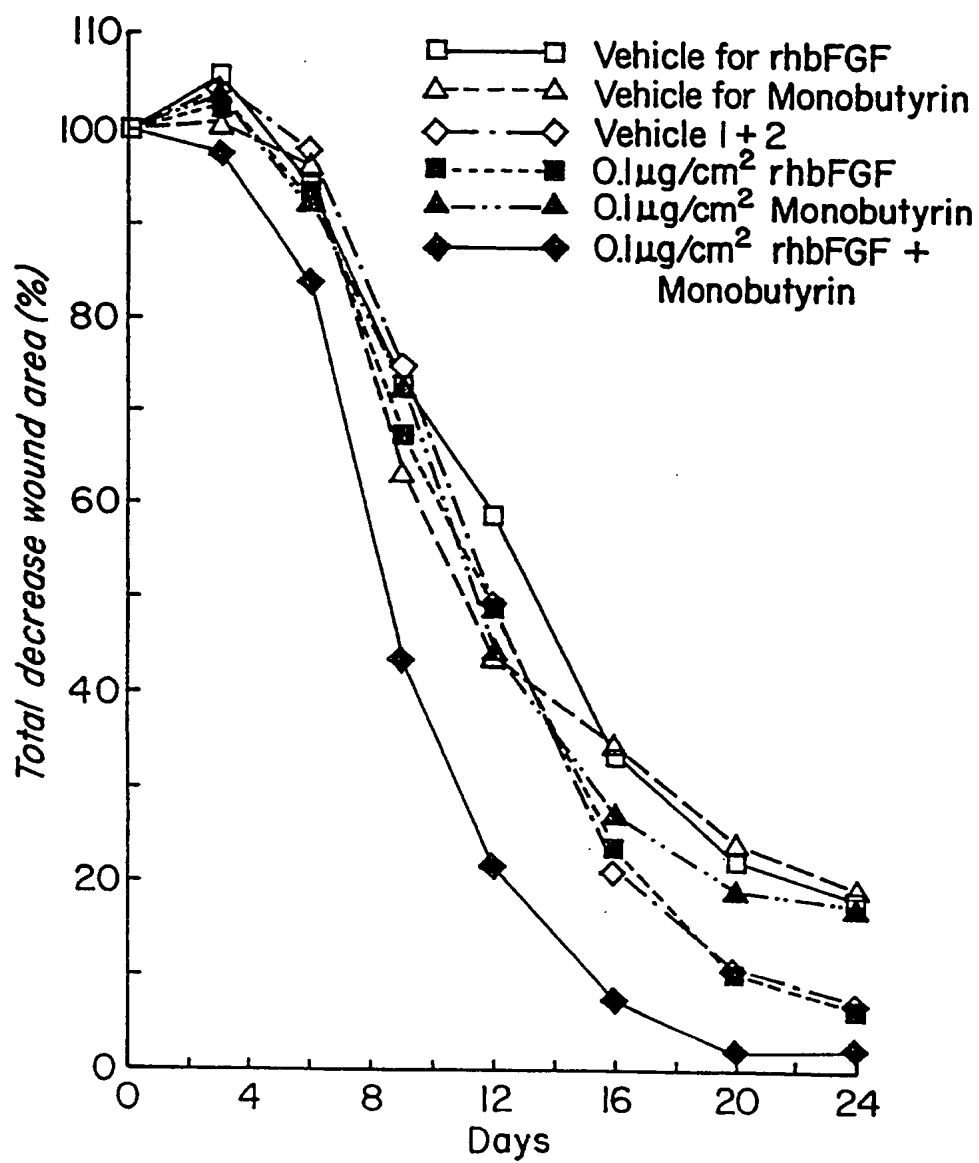


FIG. 7

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/01567

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5): A61K 31/19, 37/04		
US Cl. : 514/557; 424/85.8; 435/7; 436/501; 530/387		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
U.S.	514/557; 424/85.8; 435/7; 436/501; 530/387	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
APS, CAS, BIOSIS		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b>		
Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X, P Y, P	US, A, 4,822,821 (PERRINE) 18 April 1989, see column 3 and claim 1.	19-20 19-20
<u>X</u> Y	US, A, 4,735,967 (NEESBY), 05 April 1988, see column 4.	19-20 19-20
<u>X</u> Y	US, A, 3,952,107 (SHIBATA), 20 April 1976, see column 4.	19-20 19-20
Y	EP, A, 0 314 105 (TAKEDA CHEMICAL INDUSTRIES, LTD.), 05 March 1989, see the entire document.	1-26
A, P	US, A, 4,888,324 (CATSIMPOOLAS ET AL.), 19 December 1989, see the entire document	21-26
<p><sup>*</sup> Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
21 June 1990		06 AUG 1990
International Searching Authority		Signature of Authorized Officer
ISA/US		Jean C. Witz